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Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives

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71 **ABSTRACT**

72 The information obtained from the chemical analysis of specific human excretion products
73 (biomarkers) in urban wastewater can be used to estimate the exposure or consumption of the
74 population under investigation to a defined substance. A proper biomarker can provide relevant
75 information about lifestyle habits, health and wellbeing, but its selection is not an easy task as it
76 should fulfil several specific requirements in order to be successfully employed. This paper aims to
77 summarize the current knowledge related to the most relevant biomarkers used so far. In addition,
78 some potential wastewater biomarkers that could be used for future applications were evaluated. For
79 this purpose, representative chemical classes have been chosen and grouped in four main categories:
80 (i) those that provide estimates of lifestyle factors and substance use, (ii) those used to estimate the
81 exposure to toxicants present in the environment and food, (iii) those that have the potential to
82 provide information about public health and illness and (iv) those used to estimate the population
83 size. To facilitate the evaluation of the eligibility of a compound as a biomarker, information, when
84 available, on stability in urine and wastewater and pharmacokinetic data (*i.e.* metabolism and
85 urinary excretion profile) has been reviewed. Finally, several needs and recommendations for future
86 research are proposed.

87

88 **Key words**

89 Wastewater; Epidemiology; Biomarker; Consumption; Exposure; Population

90

91 INTRODUCTION

92 Relevant epidemiological information about lifestyle habits, public health and wellbeing can
93 be obtained from the chemical analysis of urban wastewater. This approach, called *wastewater-*
94 *based epidemiology* (WBE), is based on the analysis of specific human metabolic excretion
95 products (biomarkers) in wastewater as indicators of consumption or exposure of the population
96 served by the sewer network under investigation to different substances. WBE has been
97 successfully applied as a suitable approach for the estimation of illicit drugs consumption (Ort et al.,
98 2014; Thomaidis et al., 2016; Thomas et al., 2012; van Nuijs et al., 2011a; Zuccato et al., 2008), but
99 it has also recently been employed to assess other lifestyle-related factors such as alcohol
100 (Rodríguez-Álvarez et al., 2015; Ryu et al., 2016), nicotine (Castiglioni et al., 2015b; Lopes et al.,
101 2014; Rodríguez-Álvarez et al., 2014b), caffeine (Senta et al., 2015a) and new psychoactive
102 substances (NPS) (Kinyua et al., 2015; Reid et al., 2014a; van Nuijs et al., 2014). WBE has also
103 been applied to verify community-wide exposure to endocrine disruptors and antimicrobial agents
104 in personal care and household products (O'Brien et al., 2015; Rydevik et al., 2015). The broad
105 range of information that can be gathered from wastewater opens up the possibility of expanding
106 WBE to other human biomarkers providing clues about diet, health, diseases and exposure to
107 contaminants. For example by linking exposure to environmental or food contaminants with health
108 outcomes such as diabetes or cancer.

109 In general, a human biomarker can be an endogenous compound (produced naturally in the
110 body) or a metabolite of a xenobiotic/exogenous substance (produced through metabolic processes
111 after intentional consumption of a substance, accidental exposure to environmental contaminants, as
112 well as through diet or ingestion of a substance). Biomarkers can be classified on the basis of their
113 function as biomarkers of exposure (compounds that give information about substances consumed
114 or ingested) and biomarkers of effect (indicators of measurable changes or alterations in an
115 organism that can be associated with health problems or wellbeing) and on the basis of biological

116 nature (e.g. metabolites, hormones), or of the disease they can indicate (e.g. cardiovascular
117 biomarkers, obesity biomarkers) (Pischon, 2009).

118 The selection of a specific biomarker is not an easy task, as it needs to satisfy different
119 criteria (**Figure 1**) (Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016). From a WBE
120 perspective, a suitable biomarker must be excreted mainly via urine and concentration levels in
121 urine should be at least in the $\mu\text{g/L}$ range to ensure its detection in raw wastewater after dilution
122 (Chen et al., 2014).

123 A biomarker should also be sufficiently stable in wastewater during the transport (in-sewer
124 stability) from the input (i.e. toilet) to the sampling point and during sampling, storage and analysis
125 (in-sample stability) (McCall et al., 2016a). In wastewater biomarkers can undergo further
126 transformation due to microbial activity (Mardal and Meyer, 2014) and/or sorption to particulate
127 matter (Baker and Kasprzyk-Hordern, 2011; Daughton, 2012a; McCall et al., 2016a). The fate of
128 biomarkers in the sewer can be also predicted by using mathematical models to simulate
129 physicochemical and microbial processes (Bisceglia and Lippa, 2014; McCall et al., 2016b; Ramin
130 et al., 2016). It is important to note that biomarker transformation pathways in the sewer might be
131 different from human metabolic pathways.

132 Furthermore, a biomarker should preferably be specific to the compound under investigation
133 and unique to human metabolism, thus ensuring that its presence only derives from human
134 excretion and not from exogenous sources (Daughton, 2012b). Therefore, pharmacokinetic data on
135 human metabolism are necessary but unfortunately this information is not always feasible as for
136 many substances it is very limited or do not even exist. This information, however, is highly
137 relevant not only to back-calculate the consumption/exposure of/to a certain substance by a
138 community, but also to distinguish the amount of a substance originating from human metabolism
139 or other sources. Unfortunately, pharmacokinetic studies are time-consuming and have to fulfil
140 strict ethical rules. Alternative approaches, which allow for the identification and selection of

141 appropriate biomarkers, are therefore required; for example, *in-vitro* studies using liver enzymes,
142 which metabolize the parent compound, help in the elucidation of the chemical structure of the
143 metabolites formed (i.e. possible biomarkers) formed (Mardal et al., 2016). Computer-based *in-*
144 *silico* modelling also allow the prediction of pharmacokinetics (Reid et al., 2014a). However these
145 alternatives provide qualitative information on metabolism, but not data regarding excretion rates of
146 parent substances and their metabolites (Gracia-Lor et al., 2016).

147 The present manuscript emerges within the framework of the pan-European inter-
148 disciplinary network (Sewage analysis CORE group-SCORE), which brings together experts from
149 different disciplines interested in standardizing the WBE approach and in coordinating international
150 studies (<http://score-cost.eu/>). The aim of this review is to describe the criteria for selecting suitable
151 biomarkers and to give an overview of relevant human (urinary) metabolites and potential
152 wastewater biomarkers. Biomarkers have been grouped in four sections: (i) those that provide
153 estimates of lifestyle factors and substance use, (ii) those used to estimate the exposure to toxicants
154 present in the environment and food, (iii) those giving information about public health and (iv)
155 those used to estimate the population size. For each group and biomarker, a thorough review of the
156 available pharmacokinetic data (*i.e.* metabolism and excretion profile) and stability in urine and
157 wastewater (if known) is provided. This information can be used to evaluate their suitability
158 according to the criteria described above. Finally, potential gaps or limitations are discussed and
159 future research directions are proposed.

160

161 **2. LIFESTYLE AND SUBSTANCE USE BIOMARKERS**

162 Initially, WBE was applied to evaluate lifestyle, in particular illicit drug use within a
163 community. Its ability to deliver objective and near-real-time data on drug use, being able to detect
164 changes over time and local patterns of use, suggests that this method can be used as a
165 complementary and extended data source to existing epidemiological tools. WBE has been well

166 established for monitoring the use of cocaine, cannabis, amphetamine, methamphetamine and
167 MDMA (3,4-methylenedioxymethamphetamine).

168 Additional applications to estimate consumption of other substances, such as alcohol,
169 tobacco, caffeine and NPS, have been employed more recently. Alcohol and nicotine (tobacco) are
170 probably the most popular and accepted recreational drugs. However, many negative social,
171 economic and health aspects have been linked to their use, causing millions of deaths every year
172 (World Health Organization, 2015, 2014). It is therefore important and of particular interest for
173 policy makers to obtain continuous monitoring data on consumption levels and patterns of use, in
174 order to reduce the disease burden related to alcohol and tobacco use. Caffeine use has been
175 limitedly investigated, although it is one of the most extensively used legal stimulants, found in
176 widely-consumed products, such as coffee, tea, soft and “energy” drinks. Besides monitoring its
177 consumption, caffeine has also been proposed as a human biomarker for assessing the size and
178 dynamics of the population served (see section 5.3) by a particular wastewater treatment plant
179 (WWTP) (Senta et al., 2015a). NPS are emerging narcotic or psychotropic substances which may
180 pose similar threats to public health such as classical illicit drugs (European Union, 2005; Papaseit
181 et al., 2014). Due to the delay between their appearance on the market and their addition to the list
182 of banned (or controlled) substances, many NPS can be legally purchased, thus promoting their
183 proliferation worldwide. Furthermore, new substances appear continuously on the market (Bijlsma
184 et al., 2016; EMCDDA, 2015a). WBE has been proposed as a tool for providing useful information
185 on temporal and regional trends in the use of NPS.

186 Current state and some new features of WBE, with regard to lifestyle and substance use are
187 presented in this chapter. Furthermore, specific biomarkers of each lifestyle factor are suggested
188 (**Table S1**) and conceptual approaches for dealing with NPSs using biomarkers in wastewater are
189 proposed.

190

191 2.1. Illicit drugs

192 Among the available epidemiological indicators, general population surveys have been
193 traditionally used to assess illicit drug use at the population level. Yet, due to their inherent biases,
194 complementary and real-time approaches are needed. The determination of illicit drug consumption
195 through wastewater was first theorized by Daughton (Daughton, 2001) and implanted by Zuccato *et*
196 *al.* using cocaine as an example (Zuccato *et al.*, 2005). Since then, WBE has been widened to
197 include other illicit drugs (Asimakopoulos and Kannan, 2016; Castiglioni *et al.*, 2008; Hernández *et*
198 *al.*, 2016; van Nuijs *et al.*, 2011a).

199 The biomarkers currently used are either the illicit drug itself (i.e. amphetamine,
200 methamphetamine, and 3,4-methylenedioxy-methamphetamine-MDMA) or one of its metabolites
201 (i.e. benzoylecgonine (BEG) for cocaine, 11-nor-9-carboxy-delta9-tetrahydrocannabinol (THC-
202 COOH) for cannabis and morphine or 6-acetylmorphine for heroin).

203 Cocaine, the first substance studied in WBE, is considered unstable in wastewater; however,
204 its unique and stable metabolite (BEG) makes back-calculation to drug consumption more
205 straightforward. It must be noted that significant degradation of BEG from cocaine in sewage is also
206 reported (Plósz *et al.*, 2013), which could result in over estimation of cocaine consumption if this
207 formation is neglected. Considering human excretion rates, a cocaine: BEG ratio around 0.1 or
208 lower can indicate consumption, and any value higher (between 0.1 and 0.7) could indicate other
209 sources of cocaine, such as direct disposal (Castiglioni *et al.*, 2011a). However, more research is
210 needed in this regard (Bijlsma *et al.*, 2012; Postigo *et al.*, 2010; Van Nuijs *et al.*, 2009).

211 Δ 9-tetrahydrocannabinol (THC), the active ingredient of cannabis, is metabolized to more
212 than 20 metabolites after consumption, with 11-nor- Δ 9-carboxy-THC (THC-COOH) and 11-
213 hydroxy-THC (THC-OH) being those primarily excreted. THC-COOH has been shown to be highly
214 stable and is thus normally used to estimate cannabis consumption, albeit with some analytical

difficulties arising in multi-residue methods resulting from its non-polarity compared to other illicit drugs (Bijlsma et al., 2014; Ort et al., 2014; Pedrouzo et al., 2011).

Two more recently works studied illicit drugs are ketamine and methadone. Ketamine is a dissociative anaesthetic which has been used as a recreational drug, whilst methadone is a synthetic opioid used clinically to relieve pain and also as maintenance treatment of opioid addicts (Castiglioni et al., 2011b; Preston et al., 2003). Both ketamine and its metabolite norketamine are fairly stable in wastewater (Castiglioni et al., 2015a; McCall et al., 2016a), with the parent compound generally used as a biomarker for reliable estimation of drug usage. Variable stability for methadone has, however, been reported i.e. from high (Senta et al., 2014) to low (González-Mariño et al., 2010).

Opioids use in Europe remains a central issue, reflecting the significant impact these drugs still have on mortality and morbidity (EMCDDA, 2015b). In recent years, the production of high purity heroin has been rising, thereby increasing heroin-related mortality (UNODC, 2015). In the human body, heroin is rapidly hydrolyzed to 6-monoacetylmorphine (6-MAM) by blood esterases (Bencharit et al., 2003) and further hydrolyzed to morphine in the liver (Smith, 2009). In wastewater, heroin shows low stability (González-Mariño et al., 2010). Although 6-MAM detected in urine is used as a marker of heroin consumption (Staub et al., 2001), 6-MAM is not always detected in wastewater as it is not stable in wastewater (Thai et al., 2014). Back-calculations using 6-MAM as biomarker provides inconsistent results (Been et al., 2015). Therefore, morphine is considered as an alternative biomarker for heroin. However, therapeutic consumption of morphine should be subtracted from the total measured morphine in sewage (Khan and Nicell, 2011; van Nuijs et al., 2011a; Zuccato et al., 2016), which necessitates the availability of registered prescribed morphine at the time of wastewater sampling. Morphine is also formed in the sewer due to deconjugation of morphine glucuronide and deacetylation of 6-MAM, which imposes new

239 challenges in back-calculation schemes. Although fractions of morphine originating from codeine
240 can be considered negligible (Zuccato et al., 2008), more research is needed to find a drug
241 biomarker for heroin which fulfils all the aforementioned criteria.

242 As shown in **Table 1**, the most frequently used illicit drug biomarkers are benzoylecgonine,
243 amphetamine, methamphetamine, MDMA and THC-COOH (Thomas et al., 2012). Information
244 about excretion and stability in urine and wastewater of these and other illicit drug biomarkers less
245 frequently studied is presented in **Table S1**. One of the most current analytical challenges associated
246 with WBE is represented by chirality. Amphetamine, methamphetamine and MDMA are among the
247 illicit drugs that are chiral and as a result they can exist as enantiomers (one enantiomeric pair per
248 each chiral centre). The verification of their chiral signature in wastewater (i.e. relative proportion
249 of two enantiomers within each enantiomeric pair) allows to distinguish between illicit or licit use
250 and direct disposal (Emke et al., 2014). It has been shown that the distinction between the
251 consumption or the disposal of MDMA could be made by differentiating the loads of the
252 enantiomers present in wastewater. Indeed, enantiomeric fractions (EFs) greater than 0.5 indicated
253 illicit use, whilst EFs equal to 0.5 indicated direct disposal, when EF was calculated as follows:

$$EF = \frac{(-) - MDMA}{(-) - MDMA + (+) - MDMA}$$

255 Enantiomeric profiling of MDMA's metabolites were recently investigated in wastewater by
256 Castrignanò et al., suggesting enantioselective metabolism for HMMA (Castrignanò et al., 2016).
257 Amphetamine and methamphetamine can also be investigated at enantiomeric level, however due to
258 both legal and illicit uses, a clear understanding between consumption and direct disposal is
259 difficult (Emke et al., 2014; Kasprzyk-Hordern and Baker, 2012).

260

261 2.2. Alcohol

262 Following the consumption of alcoholic beverages, the majority of ingested ethanol is
263 rapidly metabolized in the human body in a two-stage oxidation process, first to acetaldehyde and
264 then to acetic acid. The remaining part is excreted unchanged in urine, sweat and exposed breath
265 (Jones, 1990). However, a very small fraction (<0.1%) undergoes a conjugation reaction with
266 glucuronic acid to produce ethyl glucuronide (EtG) (Dahl et al., 2002) and with 3'-
267 phosphoadenosine 5'-phosphosulfate to produce ethyl sulphate (EtS) (Helander and Beck, 2005).
268 These metabolites are excreted within a few hours and are detectable in urine for considerably
269 longer times (up to 1-2 days, depending on the subject and the alcohol dose) (Helander and Beck,
270 2005; Høiseth et al., 2008), making them unequivocal indicators of recent alcohol consumption
271 (Dahl et al., 2011; Dresen et al., 2004).

272 EtG was found to degrade ~50% after 18 hours, whereas EtS showed little or no degradation
273 (Reid et al., 2011). In addition, no significant differences were found between its stability in sewage
274 and in an ethanol-fortified wastewater sample (Reid et al., 2011), indicating that it is unlikely to be
275 formed from unconsumed alcohol discarded into the sewer system. Taking into account these
276 observations, EtS has been used by several researchers to estimate community-wide alcohol
277 consumption through wastewater analysis (**Table 1**). Typically, its determination in this matrix is
278 performed by direct injection, after filtration and/or centrifugation, into a liquid chromatography-
279 mass spectrometry system. The alcohol consumption rates estimated through WBE have revealed
280 specific drinking patterns, temporal and spatial variations. The study conducted by Reid et al. (Reid
281 et al., 2011), for example, clearly showed the weekend elevated drinking pattern in Oslo.
282 Furthermore, the estimated consumption rates were in good agreement with sales statistics (Reid et
283 al., 2011). The increase in alcohol consumption during the weekend was also found in three Spanish
284 cities, eight Belgian cities and one Italian city (Andrés-Costa et al., 2016; Boogaerts et al., 2016;
285 Mastroianni et al., 2014; Rodríguez-Álvarez et al., 2015, 2014a; Ryu et al., 2016). However, a
286 different consumption pattern was observed during a special event in Valencia, where an increased

287 alcohol use was noticeable, reaching the maximum rate on Wednesday, which corresponded to the
288 last day of the “Fallas” festivities (Andrés-Costa et al., 2016). Co-consumption of alcohol and
289 cocaine was also evaluated through WBE by analyzing cocaethylene, a specific biomarker excreted
290 when the two substances are consumed together (Mastroianni et al., 2014; Rodríguez-Álvarez et al.,
291 2015). In the studies carried out in Belgium (Boogaerts et al., 2016) and Greece (Gatidou et al.,
292 2016) higher alcohol consumption in urbanized cities than in smaller villages was evidenced.
293 Although all these studies highlight the potential of EtS as a reliable biomarker for estimating
294 alcohol consumption in relative terms, the main limitation is the uncertainty associated with its
295 percentage of excretion, which might lead to inaccurate back-calculations in absolute amounts.
296 Until now, there have been insufficient pharmacokinetic studies evaluating this percentage to
297 provide a unique, representative figure (Halter et al., 2008; Høiseth et al., 2008; Lostia et al., 2013;
298 Schneider and Glatt, 2004; Wurst et al., 2006). In the aforementioned WBE studies, the range
299 0.010-0.016% (on molar basis) was used by (Andrés-Costa et al., 2016; Reid et al., 2011); the
300 median value of the excretion rates provided by Høiseth et al. (Høiseth et al., 2008), 0.011%, was
301 used by (Mastroianni et al., 2014; Rodríguez-Álvarez et al., 2014a). Finally, four studies (Boogaerts
302 et al., 2016; Gatidou et al., 2016; Rodríguez-Álvarez et al., 2015; Ryu et al., 2016), employed a
303 people-weighted value of 0.012%, based on the data provided by (Høiseth et al., 2008) and (Wurst
304 et al., 2006).

305

306 **2.3. Tobacco**

307 Nicotine is the principal alkaloid found in tobacco and, although not being directly
308 associated with diseases, its addictiveness is the major cause of continued use of tobacco products
309 (Hukkanen, 2005). Nicotine is extensively metabolized in humans, with 70-80% of the initial dose
310 being converted to cotinine (Benowitz and Jacob, 1994), which is then further metabolized into
311 various compounds, the most abundant being *trans*-3'-hydroxycotinine (Byrd et al., 1992). Nicotine

312 and its major metabolites are also excreted as glucuronides. Globally, nicotine is excreted
313 unchanged at rates between 8 and 10%, whilst its glucuronide makes up for 3-5% of the initial dose
314 (Byrd et al., 1992). Cotinine and its glucuronide are excreted at rates between 10-15% and 12-17%,
315 respectively, while *trans*-3'-hydroxycotinine and its glucuronide make up for 33-40% and 7-9% of
316 the initial dose, respectively (Hukkanen, 2005).

317 Nicotine and its metabolites, cotinine and *trans*-3'-hydroxycotinine, have been analyzed in
318 wastewater as biomarkers (**Table S1**) to estimate tobacco use in various communities (Castiglioni et
319 al., 2015b; Lopes et al., 2014; Mackuľak et al., 2015; Rodríguez-Álvarez et al., 2014b; Senta et al.,
320 2015a). The three compounds were shown to be stable in wastewater samples stored at 4° C and 20°
321 C during 24 h (Chen et al., 2014; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). However,
322 the concentration of the glucuronide of *trans*-3'-hydroxycotinine was shown to decrease even in
323 refrigerated samples (i.e., 35% decrease over 8 h at 4° C). The authors of the study thus suggested
324 to enzymatically deconjugate the compounds prior to extraction and analysis (Rodríguez-Álvarez
325 et al., 2014b).

326 The amounts of these compounds in wastewater range from 0.1 to 7 µg/L (Buerge et al.,
327 2008; Mackuľak et al., 2015; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a), and the levels of
328 cotinine and *trans*-3'-hydroxycotinine reflected the excretion profiles expected from
329 pharmacokinetic studies, whilst nicotine was found at higher levels (Rodríguez-Álvarez et al.,
330 2014b; Senta et al., 2015a). The contribution from ashes and cigarettes butts has been advanced as a
331 possible explanation for this observation (Castiglioni et al., 2015b; Rodríguez-Álvarez et al., 2014b;
332 Senta et al., 2015a). In fact, higher nicotine levels have been reported during rain events, supporting
333 the hypothesis that ashes and cigarette butts found on streets eventually contribute to measured
334 nicotine loads (Senta et al., 2015a). Thus, cotinine and *trans*-3'-hydroxycotinine were used as
335 biomarkers to estimate the amount of nicotine used per capita in a population, as indicated in **Table**

336 1 (Castiglioni et al., 2015b; Mackul'ak et al., 2015; Rodríguez-Álvarez et al., 2014b; Senta et al.,
337 2015a).

338 In some studies, figures were corrected to account for the portion of nicotine absorbed
339 during smoking (Castiglioni et al., 2015b; Mackul'ak et al., 2015), thus providing estimates of the
340 gross amount of number of cigarettes. Additionally, Mackul'ak and co-workers (Mackul'ak et al.,
341 2015) included a factor to account for losses due to degradation, based on the mean residence time
342 of wastewater in sewers. From the estimated nicotine consumption, the number of cigarettes
343 smoked per capita was also calculated using as reference value 0.8 mg of nicotine per cigarette
344 (Gorrod and Wahren, 1993; Lopes et al., 2014; Rodríguez-Álvarez et al., 2014b) or 1.25 mg of
345 nicotine (Castiglioni et al., 2015b). The obtained figures highlighted substantial differences in
346 consumption within the same country. For example, researchers from Italy found significant
347 differences between the north, centre and south of the country (Castiglioni et al., 2015b; Senta et al.,
348 2015a). These results were in agreement with epidemiological data, which suggested a higher
349 prevalence of tobacco use in the south (Castiglioni et al., 2015b). Similarly, important differences
350 were found in cities in Slovakia and Spain (Mackul'ak et al., 2015; Rodríguez-Álvarez et al.,
351 2014b). In Portugal, estimates of nicotine consumption derived from wastewater analysis were in
352 line with findings from a European survey (Lopes et al., 2014).

353 Mass loads measured in wastewater were also used to investigate weekly consumption
354 patterns and findings suggested that this was stable throughout the week (Chen et al., 2014;
355 Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). Public holidays and specific touristic
356 locations, attracting larger crowds, were the only exceptions (Lopes et al., 2014; Mackul'ak et al.,
357 2015).

358 The results obtained show that the measurement of nicotine metabolites is a useful tool
359 which could potentially be used to complete current knowledge about the prevalence of tobacco
360 use.

361

362 **2.4. Caffeine**

363 Caffeine (1,3,7-trimethylxanthine) is the world's most widely consumed stimulating agent
364 (Garattini, 1993). It is found in many globally popular products, including tea and cola drinks, as
365 well as in some medications and dietary supplements, but the most important source of this alkaloid
366 is coffee.

367 Caffeine metabolism is extensive (Baselt, 2004), with at least 17 urinary metabolites
368 identified in humans (Garattini, 1993). The major metabolites include 1-methyluric acid (excretion
369 rate 12-25%), 1-methylxanthine (9-18%), 7-methylxanthine (2-8%), paraxanthine (1,7-
370 dimethylxanthine; 4-7%), 1,7-dimethyluric acid (5-8%) and unstable product 5-acetylamino-6-
371 formylamino-3-methyluracil (4-15%), with a small percentage (1-4%) of the initial dose excreted as
372 the parent compound (Carrillo and Benitez, 1994; Garattini, 1993). The list of caffeine metabolites
373 identified in humans, together with the excretion rates can be found in **Table S1**. Besides being
374 complex, caffeine metabolism is also rather variable, with the different excretion rates observed not
375 only in different studies, but also between individuals within the same studies (Carrillo and Benitez,
376 1994; Grant et al., 1983). These variations can be related with genetic differences (Blanchard et al.,
377 1985; Grant et al., 1983) or influenced by other factors, such as age (Blanchard et al., 1985; Grant et
378 al., 1983), pregnancy ((Carrillo and Benitez, 1994; Garattini, 1993) or medications (Callahan et al.,
379 1983). However, certain metabolites, such as paraxanthine, 1,7-dimethyluric acid and 1-
380 methylxanthine were found to be less affected by the genetic background compared to the parent
381 compound and they were, therefore, suggested as potential biomarkers for caffeine dietary intake
382 (Crews et al., 2001). Furthermore, most of the pharmacokinetic data on caffeine metabolism in
383 humans are quite old (Blanchard et al., 1985; Grant et al., 1983) and some of them include a
384 relatively low number of subjects (Blanchard et al., 1985).

385 Due to its wide usage in modern societies, caffeine is among the most ubiquitous wastewater
386 micro-contaminants, usually detected at relatively high concentration levels ($\mu\text{g/L}$) in untreated
387 wastewater (Martínez-Bueno et al., 2011; Rosal et al., 2010; Santos et al., 2009). Due to this,
388 caffeine was proposed as anthropogenic marker to indicate the discharge of domestic wastewater in
389 rivers and lakes (Buerge et al., 2003), but so far has been rarely used as a biomarker in a WBE
390 approach. Caffeine has also been proposed as a human biomarker for assessing population size and
391 the dynamics of people served by a particular WWTP (Daughton, 2012b) (see section 5.3).

392 However, with the exception of paraxanthine, data on the occurrence of caffeine metabolites
393 in wastewater are still very scarce. In fact, the first comprehensive study which included most of the
394 major caffeine metabolites (1-methylxanthine, 7-methylxanthine and paraxanthine) was published
395 just recently (Senta et al., 2015a). Concentrations of these metabolites found in Italian wastewater
396 were similar to those of the parent compound, i.e. in the $\mu\text{g/L}$ range. In the same work temporal and
397 spatial patterns of use were also studied and the mean mass loads of caffeine and its major
398 metabolites revealed to be slightly lower during the weekend, probably due to the lower
399 consumption of coffee. Similar findings for caffeine was reported by Rico et al. (Rico et al., 2016;
400 Senta et al., 2015a). On the other hand, no clear geographical trends could be observed. Besides
401 being easily detectable, caffeine, 1-methylxanthine, 7-methylxanthine and paraxanthine fulfill
402 additional important requirement for an ideal biomarker - they are stable in wastewater samples
403 stored at 4 °C and 20 °C for 24 h (Senta et al., 2015a). However, it is noteworthy that more research
404 is needed in order to select the most suitable caffeine biomarker in wastewater for the correct
405 interpretation of the obtained results within the concept of WBE.

406

407 **2.5. New Psychoactive Substances**

408 The detection of NPS and the estimation of their use are especially challenging for drug
409 epidemiology, since new compounds appear continuously on the market and consumers do not

410 always know the composition of the drugs they take. WBE can shed some light and provide
411 additional information, but it is also affected by important challenges. First, pharmacokinetic data
412 are essentially non-existent for most NPS, making it extremely difficult to define appropriate
413 biomarkers. Second, the prevalence of abuse of a single substance is generally low, leading to very
414 low concentrations in wastewater. Finally, their stability in this matrix is largely unknown
415 (EMCDDA, 2016; Reid and Thomas, 2016). Based on the limited information available, this
416 section attempts to present a selection of potential biomarkers, to be used in WBE studies, for the
417 most common classes of NPSs: synthetic cannabinoids, synthetic cathinones, phenethylamines,
418 piperazines, tryptamines, arylcycloalkylamines and benzodiazepines (EMCDDA, 2015a). The two
419 first groups constitute the largest categories and also account for the majority of seizures in Europe
420 (EMCDDA, 2015a).

421 Synthetic cannabinoids include a broad range of structurally different compounds sharing
422 affinity for the cannabinoid receptors in the brain (Pertwee, 2008). Due to their recent increased
423 popularity, their human metabolism is a growing area of research. Several *in vitro* and *in vivo*
424 experiments have been performed over the past few years and, although individual pharmacokinetic
425 profiles remain to be elucidated for many of them, it is generally thought that synthetic
426 cannabinoids are extensively oxidized in the human body and excreted as a complex mixture of
427 phase I and phase II metabolites (Fantegrossi et al., 2014; Seely et al., 2012). JWH-type
428 cannabinoids are the most popular drugs within this class. Monohydroxylation, either at the N-alkyl
429 side chain, the naphthyl moiety or the indole moiety (followed by the corresponding
430 glucuronidation) has been identified as their major metabolic pathway and, in fact,
431 monohydroxylated metabolites have been detected in urine from JWH-type cannabinoids
432 consumers (Hutter et al., 2012; Ozturk et al., 2015; Wohlfarth et al., 2013). However, the lack of
433 rigorous pharmacokinetic data, essential to calculate excretion rates, prevents from extrapolating
434 these analyses to whole communities by the WBE approach. Another important limitation concerns

435 their instability in wastewater: the scarce literature available suggests that some synthetic
436 cannabinoids and their metabolites are highly labile and tend to get adsorbed to particle matter,
437 hindering their determination and sub-estimating the potentially derived abuse calculations (Reid et
438 al., 2014a, 2014b). As a reflection of these intrinsic difficulties, to the best of our knowledge only
439 the metabolite JWH 018 N-5-hydroxypentyl and the parent compounds JWH-210 and JWH-122,
440 have been positively detected in wastewater in two out of all the studies dealing with NPS in this
441 matrix (Borova et al., 2015; Reid et al., 2014b) (see **Table S1**).

442 Synthetic cathinones are known to have been abused for approximately 15 years and the
443 synthesis of cathinone derivatives has been reported since the late 1920s (Hyde and Adams, 1928;
444 Prosser and Nelson, 2012). They all refer to cathinone ((S)-2-amino-1-phenyl-1-propanone), a
445 naturally occurring stimulant found in the leaves of *Catha edulis* (Khat) (Prosser and Nelson, 2012).
446 In general, the drugs are in part extensively metabolized in humans. However, some of the synthetic
447 cathinones are also excreted unchanged in urine (Uralets et al., 2014). Details on the metabolism
448 and detectability of synthetic cathinones can be found in original articles and are summarized in
449 several review articles (Ellefsen et al., 2015; Helfer et al., 2007; Meyer et al., 2014, 2012, 2010a,
450 2010b; Meyer and Maurer, 2010; Pawlik et al., 2012; Pozo et al., 2014; Shima et al., 2014; Staack
451 and Maurer, 2005; Uralets et al., 2014; Welter-Luedeke and Maurer, 2015). Also, data on the
452 stability, especially under storage conditions, were published (Senta et al., 2015b) and highlighted
453 the possible instability of the parent compounds under alkaline conditions (Johnson and Botch-
454 Jones, 2013; Tsujikawa et al., 2012). However, detailed and comprehensive studies are missing on
455 their chemical stability in wastewater and also biotransformation in the sewer or wastewater should
456 be considered (McCall et al., 2016a). Several studies were published on the analysis of synthetic
457 cathinones in wastewater samples, with mephedrone, methylenedioxypyrovalerone, methcathinone,
458 methylone and α -pyrrolidinovalerophenone (α -PVP) being the most frequently detected (Borova et

459 al., 2015; Chen et al., 2013; González-Mariño et al., 2016a, 2016b; Kinyua et al., 2015;
460 Mwenesongole et al., 2013; Ocaña-González et al., 2015; Thai et al., 2016; Tschärke et al., 2016).

461 Phenylethylamines are a class of substances related to amphetamine and methamphetamine,
462 possessing psychoactive and stimulant effects; however, modification of these compounds can lead
463 to potent hallucinogens (Zaitsev et al., 2011; Zawilska and Andrzejczak, 2015). They include
464 amphetamine derivatives such as MDMA, 2C and 'D' series drugs. However, the phenethylamine
465 core is shared among several compounds including cathinones and catecholamines. Several
466 metabolism studies have been conducted in an effort to understand their metabolic profiles (Ewald
467 et al., 2008, 2006; Lai et al., 2015b; Staack et al., 2003) but more information is needed.

468 Piperazine-like compounds include the original member 1-benzylpiperazine (BZP), its
469 methylenedioxy analogue and several phenylpiperazines. They are mainly known to bind to
470 serotonin receptors, with BZP additionally producing amphetamine-like stimulant effects (Bye et
471 al., 1973; De Boer et al., 2001). A summary with details on the metabolism of piperazines can be
472 found in some articles (Maurer et al., 2004; Staack et al., 2001; Staack and Maurer, 2005);
473 furthermore, one study showed the detection of metabolites in human urine (Tsutsumi et al., 2005).
474 Some examples are shown in **Table S1**.

475 Tryptamine is a primary amine alkaloid found widely in nature in both the plant and animal
476 kingdoms and known for its hallucinogenic effects (Collins, 2011). Metabolism of some synthetic
477 tryptamines has been studied (Kamata et al., 2006; Michely et al., 2015; Narimatsu et al., 2008).

478 Arylcycloalkylamines, which include the ketamine derivative methoxetamine (MXE) and
479 phencyclidine derivatives, have emerged as legal alternatives to ketamine (Roth et al., 2013). MXE,
480 which has gained popularity in several European countries (EMCDDA, 2014), is extensively
481 metabolized (Meyer et al., 2013) but it was detected as parent MXE in wastewater from Belgium
482 and Switzerland (Kinyua et al., 2015).

483 Benzodiazepines are psychoactive substances whose core structure is a benzene ring fused
484 to a diazepine ring. Benzodiazepines are known as tranquilizers and are among the most commonly
485 prescribed antidepressant medications. Although a useful pharmaceutical, there is potential for
486 abuse due to their hypnotic and sedative effects – even to the extent of being used as “date rape”
487 drugs (Schwartz et al., 2000). From now on we will refer to those benzodiazepines used illegally as
488 design benzodiazepines. Designer benzodiazepines have become a rapidly growing class of drugs
489 on the NPS online market, since a medical prescription is not needed. Since designer
490 benzodiazepines have increased in popularity, studies have been conducted characterizing their
491 human metabolism (Huppertz et al., 2015; Moosmann et al., 2013).

492 Up to now, no designer phenethylamines, tryptamines or designer benzodiazepines and
493 metabolites have been detected in wastewater and only two studies have reported the stability of
494 some phenylethylamines in wastewater (Bade et al., 2016; Senta et al., 2015b).

495 Although the interpretation of quantitative results should be done carefully for NPS due to
496 the lack of metabolic information, the qualitative monitoring could lead to a better understanding of
497 the frequency of use and could identify changes in consumption.

498

499 **3. EXPOSURE BIOMARKERS FROM ENVIRONMENT AND FOOD**

500 Two important exposure pathways for potentially harmful compounds are the dietary intake
501 and the exposure from the surrounding daily environment. The monitoring of various classes of
502 compounds for which exposure commonly occurs through these routes is necessary to safeguard
503 public health. Representative chemical classes have been chosen as examples for this paper.
504 Pesticides, mycotoxins and parabens are three classes of compounds for which exposure occurs
505 through the intake of contaminated food or absorption through the skin and adverse health effects
506 can be foreseen for humans (Błędzka et al., 2014; Heyndrickx et al., 2015; Rizzati et al., 2016;
507 Warth et al., 2013). Exposure through the indoor environment (furniture, electronics, packaging and

508 personal care products (PCPs)) is characteristic for UV-filters, plasticizers and brominated flame
509 retardants.

510 This section reviews the specific biomarkers of each of the above mentioned chemical
511 classes which could be measured in wastewater in order to assess the overall exposure to these
512 compounds through a WBE approach. When relevant, we have also included the metabolites of
513 these chemicals to be explored as a suitable biomarker. The suggested biomarkers are reported in
514 **Table S2** including also metabolites, whenever such information is available.

515

516 **3.1 Pesticides**

517 Pesticides are chemicals commonly used for control of harmful organisms, such as fungi,
518 insects and weeds. They are mostly used for crop protection, but can also be used for livestock
519 protection, as well as for other industrial and household purposes, such as termite prevention. The
520 general population is exposed to pesticides mainly through diet (Ntzani et al., 2013), but also
521 through household use (Trunnelle et al., 2013) and inhalation of polluted air - particularly in
522 agricultural areas where aerial spraying of pesticides occurs (Coscollà et al., 2010). Exposure to
523 pesticides is of public concern as they may cause health effects such as elevated rates of chronic
524 diseases, like cancer or diabetes, as well as neurodegenerative disorders such as Parkinson disease,
525 birth defects and reproductive diseases (Rizzati et al., 2016). Young children are the most
526 susceptible to be at risk (European Food Safety Authority, 2013).

527 There are several types of pesticides and they are generally classified by their chemical
528 structure: carbamate, organophosphate or triazine pesticides (**Table S2**). They may also be
529 classified by the type of pest they control, such as herbicides, which are intended to kill weeds and
530 other unwanted plants, and insecticides, which kill insects and other arthropods. Pesticides are
531 mostly formulated as mixtures with individual components which may act independently of each
532 other, interact or have dose-addition effects (Hernández et al., 2013).

533 Until now, there are only two WBE studies (Rousis et al., 2016a, 2016b) published on
534 human exposure to pesticides. The first work (Rousis et al., 2016a) proposed for the first time a new
535 application for pesticides, where pyrethroid, triazine and organophosphate metabolites were
536 monitored in influent wastewater of seven Italian cities. The most frequently detected compounds
537 were the specific metabolite of chlorpyrifos and chlorpyrifos-methyl, 3,5,6-trichloro-2-pyridinol
538 (TCPY), the metabolite of diazinon (2-isopropyl-6-methyl-4-pyrimidinol, IMPY), the pyrethroid
539 metabolites 3-phenoxybenzoic acid (3-PBA, common metabolite of about 20 pyrethroids), 3-(2,2-
540 dichlorovinyl)-2,2-dimethyl-(1-cyclopropane)carboxylic acid (DCCA, common metabolite of
541 permethrin, cypermethrin and cyfluthrin) and two alkyl phosphate metabolites. The second work
542 (Rousis et al., 2016b) applied the novel WBE approach to assess further exposure to pyrethroids,
543 concretely 3-PBA, cis-DCCA and trans-DCCA. The obtained results were in agreement with the
544 Human Biomonitoring (HBM) profiles in urine samples of the general population, reported in the
545 literature.

546 Yusa et al. 2015 reviewed analytical methods for HBM of pesticides and found that the most
547 commonly biomonitored ones are carbamates, herbicides, neonicotinoids, organophosphates,
548 pyrethroids and sulfonylurea herbicides – all of which can be monitored in urine samples and they
549 can be good potential biomarkers for WBE. However, some other pesticide classes, such as
550 organochlorines, are probably not suited to WBE due to their non-polar characteristics and their
551 poor excretion in urine (Yusa et al., 2015).

552 As described previously for other substances, the metabolites of pesticides rather than the
553 parent substances should be measured in wastewater to avoid contributions from sources other than
554 human metabolism. It has to be emphasized that some pesticide metabolites are also formed in the
555 environment (i.e. atrazine undergoes dealkylation in water systems forming human metabolites) and
556 therefore more research is needed. Moreover, there are some common metabolites produced by

different classes of compounds, such as organophosphate pesticides, organophosphate plasticizers and flame retardants, and this should be taken into account in a WBE approach. The novel method developed by Rousis et al. is considered as a valuable tool for obtaining objective, direct information on pesticide exposure levels and could provide complementary information for HBM studies. **Table S2** presents the main potential biomarkers of exposure to pesticides selected by considering the detection frequency in urine, and the concentration levels (Barr, 2008; Yusa et al., 2015).

3.2 Mycotoxins

Mycotoxins are toxic fungal metabolites that can be found in food and feed which are intended for human and animal consumption (i.e. cereals such as rice, maize and wheat). There is huge concern of human health risks related to the ingestion of these substances, since they are stable in food processing and cooking. Maximum tolerable levels in food commodities were therefore legally established in many countries (*Commission Regulation 1831/2003*, 2003). While, nowadays, approximately 400 compounds belong to this group, only 10-15 are considered to be priority mycotoxins, due to higher occurrence and toxicity. These latter compounds belong to the groups of aflatoxins, ochratoxins, patulin and fusarium toxins (tricothecenes, fumonisins, zearalenone and zearalenone derivatives) (Anfossi et al., 2016; Turner et al., 2015). HBM studies performed on general population have shown that the most studied mycotoxin biomarkers in urine samples are aflatoxin M1 (AFM1), ochratoxin A (OTA), deoxynivalenol (DON), nivalenol (NIV), fumonisin B1 (FB1) and zearalenone (ZON) (H Fromme et al., 2016; Heyndrickx et al., 2015). If mycotoxin contaminations are going to be increased in the near future due to higher global food demand and global climate and environment changes, new methods are needed to evaluate the human exposure

580 to mycotoxins (Marroquín-Cardona et al., 2014). Thus, a novel approach such as the WBE can be
581 useful to provide complementary information to existing methods.

582 Few studies dealing with the determination of mycotoxins in wastewater have been
583 published. The studied analytes were detected at very low concentrations (few ng/L), but at high
584 detection frequency. In addition to parent compounds, some human metabolites were also
585 investigated. The detected mycotoxins were DON, beauvericin (BEA), 3-Acetyldeoxynivalenol (3-
586 AcDON), NIV, ZON, α -zearalenol (α -ZOL) and β -zearalenol (β -ZOL) (Kolpin et al., 2014; Laganà
587 et al., 2004; Schenzel et al., 2012, 2010; Wettstein and Bucheli, 2010). None of these studies
588 attempted to apply the WBE approach to these substances; they had only a monitoring scope. In the
589 present paper a selection of mycotoxins and their related potential biomarkers for a WBE approach
590 were reported for the first time (**Table S2**).

591

592 **3.3 Parabens**

593 Parabens are a group of chemicals that is drawing a lot of interest in the current discussion
594 given their potential endocrine disrupting properties, since studies have shown that they have
595 potential adverse health effects (Hu et al., 2013; Kim et al., 2015; Zhang et al., 2013). This has
596 raised concern considering their widespread use. Parabens are used as preservatives in many
597 different products, such as cosmetics, PCPs and foods, and can be commonly found in household
598 products.

599 Some studies also investigated the occurrence and fate of parabens in wastewater (González-
600 Mariño et al., 2009; Gracia-Lor et al., 2012a; Kasprzyk-Hordern et al., 2008), but not from a WBE
601 perspective. Therefore, a list of known urinary biomarkers for paraben exposure is reported in
602 **Table S2**. Future research should be addressed in order to explore paraben biomarkers for WBE.

603

604 **3.4. UV-Filters**

605 Overexposure to ultraviolet (UV) radiation has been associated with skin disorders, such as
606 cancer (Ramos et al., 2016). This led to the widespread usage of UV filters in a variety of personal
607 care products to protect against UV radiation, i.e., sunscreen, cosmetics, beauty creams, body
608 lotions, hair sprays and shampoos (Brausch and Rand, 2011). UV filters are also used in food
609 packages, plastics and textiles to prevent polymer degradation. Hence, human exposure occurs
610 through multiple routes such as dermal absorption, ingestion of contaminated food and tap water
611 (Valle-Sistac et al., 2016). Two major types of UV filters are currently available; organic UV filters
612 are used to absorb UVA and/or UVB radiation, whereas inorganic UV filters mainly reflect the
613 radiation. Given the high photostability and lipophilicity, many UV filters can enter biological
614 membranes and bioaccumulate in the body, including in the placental tissues (Valle-Sistac et al.,
615 2016). However, it is important to note that most UV-Filters are released into the sewers without
616 going through the body (Daughton and Ruhoy, 2009; Ruhoy and Daughton, 2008). This fact would
617 contribute to a large uncertainty in its estimation.

618 Urinary analysis has frequently detected UV filters at various levels, demonstrating human
619 exposure (Dewalque et al., 2014; Louis et al., 2015). Despite their widespread use, between 2010
620 and 2015 only 20 studies have been published in peer reviewed journals dealing with UV filters
621 detection in wastewater (Ramos et al., 2016). Yet, available data indicates that major UV filters
622 groups, i.e. benzophenone derivatives, p-aminobenzoic acid derivatives, camphor derivatives,
623 benzotriazole derivatives, salicylate derivatives, benzimidazole derivatives, triazine derivatives,
624 cinnamate derivatives, crylene derivatives, and dibenzoyl methane derivatives, are ubiquitous in
625 wastewater with concentrations ranging from the ng/L to the mg/L level (Gago-Ferrero et al., 2011;
626 Rodil et al., 2012). Evidence from mammalian studies indicate that various UV filters are endocrine
627 disruptors, acting as estrogenic, antiestrogenic, antiandrogenic or antithyroid (Louis et al., 2014).
628 These results find support in recent epidemiologic studies reporting an association between human
629 urinary levels of certain UV filters and couples fecundity, i.e. BP-2 (Louis et al., 2014), and

630 decrease semen quality, i.e. BP-3 and BP-8. Therefore, (Louis et al., 2015) highlighted the
631 importance of further studies exploring human exposure to UV filters. Despite the presence of UV
632 filters has been reported in wastewater (Ramos et al., 2016; Tsui et al., 2014) no WBE approaches
633 have been yet tested to evaluate human exposure to these substances. However, the high stability of
634 these compounds and the indication of particular metabolite signatures (Le Fol et al., 2015) suggest
635 potential biomarkers for UV filters in wastewater based biomarkers to support epidemiological
636 studies (**Table 1 and S2**).

637

638 **3.5. Plasticizers**

639 Plastics are very versatile materials typically consisting of organic polymers of high
640 molecular mass, which may contain other substances. Manufacturers often add different chemicals
641 to plastics to give them specific characteristics, such as flexibility, resilience and pliability. These
642 plasticizers mainly include phthalates and adipates, and because of their environmental persistence
643 and their widespread use, it is unsurprising that they can be found in wastewater and in the
644 receiving environment (Barnabé et al., 2008; Gao and Wen, 2016; Olofsson et al., 2013; Zolfaghari
645 et al., 2014). Some of these chemicals and/or their derivatives interfere with endogenous hormone
646 signalization in animals and humans, raising concerns about their potential to cause long-term
647 diseases (Joint Fao Oms Expert Committee On Food Additives, 2010). In particular phthalates (e.g.
648 bis(2-ethylhexyl) phthalate and, dibutyl phthalate) were associated with the disruption of
649 hormonally-mediated pathways, as well as increased risk for cancer (“Toxicological profile for
650 di(2-ethylhexyl)phthalate (DEHP),” 2002, “Toxicological profile for Di-n-butyl-Phthalate,” 2001).
651 Furthermore, epidemiological observational studies suggest that there is a consistent association of
652 blood and urine concentrations of phthalates, and some effects, such as those mentioned above
653 (Joint Fao Oms Expert Committee On Food Additives, 2010; Kim et al., 2015; Wang et al., 2016).
654 Due to a better toxicological profile (Bhat et al., 2014) and a better blood compatibility (Zhong et

655 al., 2013), other plasticizers, such as di-isononyl cyclohexane-1,2-dicarboxylate (DINCH), have
656 been increasingly used in recent years as alternatives in PVC films and medical devices.
657 Metabolites of phthalates, adipates, and DINCH have been found in urine (Fromme et al., 2016;
658 Guo et al., 2011; Herrero et al., 2015; Loftus et al., 1993; Silva et al., 2007), but their presence in
659 wastewater has never been investigated. For a list of known biomarkers in urine see **Table S2**.

660

661 **3.6 Flame retardants**

662 Flame retardants (FRs) are chemical additives for manufactured materials, such as plastics
663 and textiles, to inhibit, suppress, or delay the production of flames to prevent the spread of fire.
664 Brominated flame retardants (BFRs) and organophosphorus flame retardants (PFRs) are the most
665 used classes of organic FRs. Due to their high log K_{ow} , BFRs are lipophilic and preferentially
666 retained in the human body, e.g. in the blood or adipose tissue. They are only slowly metabolized to
667 hydroxylated metabolites (e.g. HO-PBDEs), which are also retained in the body and thus not
668 excreted in the urine. The presence of BFRs in the sewer system is largely due to direct input from
669 the indoor environment, following washing out of dust and being associated with particles. PFRs
670 are less persistent and rapidly metabolized in the human body (Van den Eede et al., 2013), they
671 have been measured in municipal wastewater in Europe (Loos et al., 2012; Marklund et al., 2005),
672 Australia (O'Brien et al., 2014) and United States (Schreder and La Guardia, 2014). PFRs
673 metabolites are excreted via urine and they are thus suitable biomarkers to assess human exposure
674 to PFRs (Van den Eede et al., 2015); however, there are no reports on the presence of PFR
675 metabolites in wastewater and no studies testing them in a WBE approach (**Table S2**).

676

677 **4. HEALTH BIOMARKERS**

678 Community health programs play an essential role for public health agencies to monitor and
679 evaluate the present status of health in a community and measure the success of programs aimed at

680 improving it. Current challenges mainly consist of the quick and reliable evaluation of the overall
681 health of a population, and detect possible health and illness threats such as pandemics or higher
682 prevalence of diabetes or cancer.

683 The quantitative measurement of specific exogenous and endogenous biomarkers related to
684 these diseases in wastewater has the potential to provide rapid information on different factors
685 related to public health and illness. Specific classes of pharmaceuticals such as antibiotics and
686 benzodiazepines and their metabolites are exogenous compounds, which can be related to their use
687 for specific illnesses or diseases, whereas endogenous compounds, such as α -fetoprotein,
688 choriongonadotropin (hCG) and isoprostanes, are more directly related to cancer or stress.

689 In this section, both exogeneous and endogenous specific biomarkers are presented and
690 suggested to monitor health issues (**Table S3**) through the WBE approach. In addition, DNA-based
691 approaches, currently applied in the field of WBE, have been reviewed.

692

693 **4.1 Pharmaceuticals**

694 **4.1.1 Antibiotics**

695 Antibiotics (ABs) can be suitable biomarkers for representing human health status
696 associated with bacterial infections. The determination of reliable data on their consumption is of
697 interest as AB use is one of the main factors responsible for AB resistance (Euro-CDC, 2012). WBE
698 may give a better understanding of real time use and misuse of ABs at the population level, by
699 supporting for example prescription data from official sources and annual sales.

700 Many ABs are excreted unchanged in urine (Castiglioni et al., 2006; Huang et al., 2011),
701 hence, parent drugs are generally targeted as biomarkers (**Table S3**). However, the selection of a
702 significant AB biomarker should not be limited to the parent drug only; in fact, the investigation of
703 specific metabolites is adding specificity to the analysis avoiding biases coming from the direct
704 disposal of the AB. This is particularly relevant for ABs widely used for veterinary treatments. The

705 most targeted classes of ABs are β -lactams, quinolones and fluoroquinolones, sulphonamides,
706 tetracyclines and macrolides. Apart from β -lactams that undergo easy hydrolysis, sulphonamides
707 and macrolides are very persistent, and are therefore also detected in treated wastewater (Jelic et al.,
708 2012). Stability of the ABs metabolites in wastewater is less understood.

709 The occurrence of ABs in influent wastewater has been widely investigated in several
710 countries (Gracia-Lor et al., 2012b; Kümmerer, 2009; Verlicchi et al., 2012). Seasonal variability of
711 population-normalized mass loads was observed by Castiglioni et al. 2006, using the WBE
712 approach, showing a difference in percentage from winter to summer of 47, 77 and 100 for
713 ciprofloxacin, ofloxacin and sulphamethoxazole, respectively (Castiglioni et al., 2006). Temporal
714 monitoring of ABs at several time scales showed a higher variability monthly/hourly than
715 daily/weekly along with seasonality in mass fluxes for ciprofloxacin, ofloxacin and clindamycin
716 (Coutu et al., 2013). Deconjugation during in-sewer transport may influence the influent loading of
717 sulfamethoxazole (Snip et al., 2016) depending on the type and size of the served catchment
718 (Polesel et al., 2016). Application of WBE helped in determining the usage of ABs in areas where
719 consumption data were scarce or a proper regulation was missing, revealing an excessive use in
720 China (Yuan et al., 2015).

721

722 **4.1.2 Benzodiazepines**

723 Benzodiazepines are used therapeutically for a considerable number of applications,
724 including anxiety and sleep disorders. Their primary mode of action is an enhancement of the action
725 of the neurotransmitter gamma-aminobutyric acid which may result in anticonvulsant, anxiolytic,
726 hypnotic, muscle relaxant and sedative effects. Benzodiazepines and benzodiazepine analogs are
727 commonly prescribed; however, they are also among the most frequently abused prescription
728 medications (Button, 2015). Despite the risk for abuse, approximately 5.2% of US adults between

729 18 and 80 years of age used benzodiazepines in 2008, with a double prevalence for women than
730 men (Olfson et al., 2015). As such, monitoring of benzodiazepines is of public concern.

731 Monitoring benzodiazepines in populations could be achievable via WBE as they are
732 normally halogenated and hence resistant to biodegradation (Kosjek et al., 2012). Multiple studies
733 have already identified both parent benzodiazepines and their urinary metabolites in wastewater
734 influent (Baker et al., 2014; Borova et al., 2014; Castrignanò et al., 2016; Fernández et al., 2014;
735 Hummel et al., 2006; Kosjek et al., 2012; Racamonde et al., 2015, 2014). Differences in the
736 behavior of benzodiazepines are associated with differences in functional substituent groups, and
737 mainly the hydroxylated tranquilizers, oxazepam, and temazepam, were reported to be present in
738 influent and effluent wastewater (Bijlsma et al., 2012; Hummel et al., 2006; Löffler et al., 2005).

739 A summary of the most commonly prescribed and detected benzodiazepine parent
740 compounds and their metabolites, which have been identified in urine, in addition to identification
741 in wastewater and stability data, when available, are presented in **Table S3**.

742

743 **4.1.3 Other pharmaceuticals**

744 Even if many works have analysed the presence of pharmaceuticals in urban wastewater,
745 only a few studies investigated these chemicals as WBE biomarkers. Some examples can be found
746 in **Table 1**. Furthermore, a list of proposed pharmaceuticals is given in **Table S3** with their
747 excretion rates.

748

749 **4.1.4. Chiral pharmaceuticals**

750 More than 50% of pharmaceuticals currently used are chiral although they are usually
751 manufactured as racemic mixtures (Petrie et al., 2015; Vazquez-Roig et al., 2014). Human
752 metabolism and microbial processes during wastewater treatment can result in the enrichment of
753 one specific enantiomer. Thus, the analysis of chiral compounds in wastewater allows to distinguish

754 between usage of pharmaceuticals due to intentional human ingestion and from accidental release
755 (direct disposal). For instance, enantioselective analysis was used by (Vazquez-Roig et al., 2014) to
756 tentatively propose direct disposal of atenolol where a moderate higher average daily load was
757 observed. Recently, (Petrie et al., 2016) identified direct disposal of the antidepressant fluoxetine
758 via the sewer network using wastewater analysis.

759

760 **4.2. Endogenous compounds**

761 Endogenous chemicals are produced by biological processes associated with stress or
762 normal metabolism. Changes in biological mechanisms may result in alterations of the endogenous
763 compound production and, therefore, measurement of such compounds can be used as indicator of
764 health status and disease (Daughton, 2012b; Group, 2001; Hagger et al., 2006). Endogenous
765 biomarker analysis has been extensively studied as diagnostic or prognostic tools in clinical
766 medicine, and can be further applied to the field of WBE (Daughton, 2012b). Thus far, the
767 investigation of endogenous biomarkers has been more focused on diseases such as cancer, diabetes
768 and cardiovascular disorder than on the overall health status. However, the number of biomarkers
769 validated for routine clinical practice is rather limited (Poste, 2011; Rifai et al., 2006), which falls
770 into even smaller numbers of biomarkers for WBE when considering only those excreted into urine.
771 Nevertheless, a range of endogenous compounds have been suggested as wastewater biomarkers of
772 effect including cancer (prostate specific antigen, α -fetoprotein) (Thomas and Reid, 2011; Yang et
773 al., 2015c), oxidative stress (isoprostanes) (Daughton, 2012b; Ryu et al., 2015; Thomas and Reid,
774 2011) and health (anti-inflammatory eicosanoids) (Daughton, 2012b). To date, studies conducted on
775 candidate endogenous biomarkers in wastewater are based on targeted analysis of specific markers
776 such as isoprostanes (Ryu et al., 2015) and cancer biomarkers (Yang et al., 2015c). However, it is
777 important to note that omics approaches also hold promising and important roles in future

778 developments and applications of endogenous biomarkers analysis in WBE (Rice et al., 2015). The
779 added value of analyzing these compounds would reside mainly in relative comparisons, both intra-
780 and inter- communities (Daughton, 2012b). Compared to the interpretation of the exogenous
781 biomarkers, where absolute values are emphasized, the use of endogenous biomarkers is more
782 focused on detecting changes over time or between communities. Such data can reveal emerging
783 trends (i.e., early warning system) and health disparities caused by various factors (e.g., exposure,
784 lifestyle).

785

786 **4.3. DNA**

787 The demand for sensitive, low-cost and high-throughput methods to characterize DNA/RNA
788 sequences has driven the development of molecular biology techniques and bioinformatics, i.e.,
789 PCR-based approaches and next generation sequencing (NGS) (Ryoo et al., 2013). Massive
790 sequencing is nowadays possible, owing to the development of different NGS platform that allows
791 an entire genome to be sequenced in less than one week. These technical advances led to a rapid
792 increase in new applications, including DNA-based health biomarkers. During the last decade an
793 increasing number of studies took advantage of these developments, and applied them to the field of
794 WBE. Several examples highlight the potential of the approach. In the field of virological
795 surveillance, wastewater screening has been used to identify the viral strains that are circulating in
796 the community, supporting epidemiological studies of the related viral infections and working as an
797 early warning tool (Hellmér et al., 2014; Kokkinos et al., 2011; Mclellan et al., 2013; Zhou et al.,
798 2014). Hellmér et al. 2014 investigated the presence of eight pathogenic viruses (norovirus,
799 astrovirus, rotavirus, adenovirus, Aichi virus, parechovirus, hepatitis A virus [HAV], and hepatitis E
800 virus) in wastewater from Sweden to explore whether their identification could be used as an early
801 warning of outbreaks. Results show that two strains were involved in an ongoing outbreak in

802 Scandinavia and were also identified in samples from patients with acute hepatitis A in Gothenburg
803 during spring of 2013.

804 A similar framework has been applied in other areas such as the study of the epidemiology
805 of the emerging human pathogens (McLellan et al., 2013; Webb et al., 2015), and antibiotic
806 resistance patterns of populations (Colomer-Lluch et al., 2014; Kumaraswamy et al., 2014;
807 McLellan and Eren, 2014). One of the most recent applications has been in the field of human
808 metabolic disorders. With the obesity epidemic reaching alarming levels, there is a need to set
809 biomarkers to identify populations or sub-populations at risk (Lyssimachou et al., 2015). Recently,
810 a good correlation has been established between the gut microbiome and obesity. In fact, only a few
811 bacterial species are sufficient to distinguish between lean and obese individuals (Le Chatelier et al.,
812 2013). These findings prompted a large study in the US using oligotyping of high-throughput 16S
813 rRNA gene sequence data to screen wastewater from 71 cities. It was demonstrated that cities could
814 be differentiated by their sewage bacterial communities, and the community structures were good
815 predictors of a city's estimated level of obesity (Newton et al., 2015). This example illustrates that
816 once specific biomarkers are identified, DNA-based analysis in wastewater can work as a powerful
817 tool to support epidemiological studies

818

819 **5. POPULATION BIOMARKERS**

820 Accurate estimation of population size is necessary to normalize WBE data to the per capita
821 level, which allows for temporal and spatial comparisons to be made (van Nuijs et al., 2011b). A
822 review of all uncertainties associated with WBE found that there is a direct relationship between the
823 uncertainty in measuring the population size and the uncertainty in the calculated daily loads of
824 drugs (Castiglioni et al., 2013; Lai et al., 2015a). Therefore, accurate data on population size are
825 needed to make decisions involved with planning and forecasting, assessing services and

826 infrastructure, policy making, informing legislation and resource allocation at the level of
827 neighborhood, city, province or country.

828 Current methodologies to estimate population size are based on public surveys (such as
829 census taking), complemented with a wide array of demographic statistics, such as tourism and
830 potential commuters. Census, however, can become increasingly outdated and cannot be easily
831 updated to accommodate change such as births, deaths, and migration (movement). Ideally, the
832 census should be able to estimate both the *de jure* and the *de facto* population. The *de jure*
833 population comprises all “usual” residents, mainly those with formal residences. The *de facto*
834 population comprises all those who are present, regardless of the location of their formal or usual
835 residence (Daughton, 2012a). A *de facto* population therefore includes all non-residents (e.g.,
836 commuters, visitors, tourists) and excludes all permanent residents who are absent. However, the
837 census approach acquires a static snapshot estimate and usually succeeds in only capturing a portion
838 of the population. Population size can also be estimated from hydrochemical parameters that are
839 routinely determined in the WWTPs, including chemical oxygen demand (COD), biological oxygen
840 demand (BOD) and total nitrogen and phosphorus. However, these parameters are highly influenced
841 by wastewater composition (i.e. industrial, domestic or mixed).

842 Addressing the population uncertainty and identifying suitable markers for the population
843 size markers is thus an important aspect of WBE (Been et al., 2014; Brewer et al., 2012; Lai et al.,
844 2011; O’Brien et al., 2014). Many compounds can be considered as biomarkers for population size.
845 Possible candidates are both naturally occurring and synthetic xenobiotics (and their metabolites or
846 formulation impurities), as well as products of endogenous metabolism. A variety of chemicals
847 have been studied as biomarkers of population, including drugs (e.g., carbamazepine (Gasser et al.,
848 2010)), biocides (e.g., triclosan (Singh et al., 2010)), chemicals in household cleaning agents, e.g.,
849 fluorescent whiteners, trialkylamines (Managaki et al., 2006; Valls et al., 1989), and food additives,
850 e.g., sucralose (Oppenheimer et al., 2011). An essential characteristic for a biomarker to be useful

851 for measuring population size is, in addition to the general requirements for a biomarker, to have a
852 low variance in the per capita daily excretion (Daughton, 2012a); the knowledge of quantities
853 excreted daily ensures that diurnal variations (e.g., resulting from circadian biorhythms) are fully
854 accommodated. Another requisite for these groups of biomarkers is that daily per capita excretion
855 should not be affected by variables such as season, weather and geographic location.

856 To date, none of the population size markers proposed have yet met all necessary criteria
857 mentioned above and additional characteristics described before for a WBE biomarker should also
858 be considered. Some specific applications are listed below.

859

860 **5.1 Artificial sweeteners**

861 The most popular artificial sweeteners used in foodstuffs include acesulfame (ACE), alitame
862 (ALI), aspartame (ASP), cyclamate (CYC), neotame (NEO), neohesperidin dihydrochalcone
863 (NHDC), saccharin (SAC) and sucralose (SUC) (**Table S4**) (Kokotou et al., 2012; Lange et al.,
864 2012). All of them, except NEO and ALI, are allowed to be used as additives in food by the
865 European Union (EPCD, 2003), whereas five of them, ACE, ASP, NEO, SAC and SUC are
866 approved to be used in the United States (USFDA, 2006).

867 After ingestion, ACE, CYC and SAC are unaffected by the human metabolism, and thus
868 largely eliminated from human bodies mainly unchanged in urine (Fermin and Vallvey, 2004;
869 Lange et al., 2012; Renwick, 1985; Roberts et al., 2000; Sardesai and Waldshan, 1991). Studies
870 have shown that, due to variations in individual metabolism, CYC could be metabolized to
871 cyclohexylamine and excreted in urine (Renwick et al., 2004). For ALI, 7–22% is excreted
872 unchanged in feces, while the rest, about 78–93% is hydrolyzed to aspartic acid and alanine amide
873 (Fermin and Vallvey, 2004). The glucuronide conjugates of ALI metabolites are the major urinary
874 metabolites in the first 24 hours. ASP is largely broken down in human gut to aspartic acid,
875 phenylalanine and methanol (Fermin and Vallvey, 2004; Lange et al., 2012). NEO and its

876 metabolites are excreted in urine and feces (WHO Food Additive Series No. 52, 2004). Less than
877 2% is excreted unchanged, but it is extensively metabolized in humans via de-esterification to *N*-[*N*-
878 (3,3-dimethylbutyl)-*L*-*alpha*-aspartyl]-*L*-phenylalanine (WHO Food Additive Series No. 52, 2004).
879 Minor metabolites of NEO include *N*-(3,3-dimethylbutyl)-*L*-aspartic acid, 3,3-dimethylbutanoic
880 acid and the carnitine conjugate and glucuronide conjugate of 3,3-dimethylbutanoic acid (WHO
881 Food Additive Series No. 52, 2004). NHDC is hydrolyzed in humans to isoferulic acid, 3-
882 hydroxyphenylpropionic acid, and 3-hydroxycinnamic acid (Fermin and Vallvey, 2004; Lange et
883 al., 2012). SUC is mainly excreted unchanged in human feces, while 8-22% was excreted in urine
884 unchanged together with its glucuronide conjugates (Roberts et al., 2000).

885 ACE, CYC, SAC, and SUC were found highly stable in raw wastewater at 4°C and room
886 temperature over four days (Ordóñez et al., 2012). Under these conditions, only 20-30% of ASP
887 remained after one day and none left after two days. Similarly, the amount of NHDC was found less
888 than 10% in the raw wastewater at 4°C after one day and linearly decreased at room temperature
889 over three days. Similar results were also reported in another study, in which ACE, CYC, SAC and
890 SUC remained stable in raw wastewater at 4°C over three weeks, whereas ASP and NHDC were
891 degraded within a day (Tran et al., 2013).

892 Since they are exclusively non-metabolized in humans and highly stable in wastewater, the
893 parent compounds ACE, CYC, SAC and SUC can be measured for the WBE approach. However,
894 the analysis of the metabolites of ALI, ASP, NEO and NHDC, rather than of the parent compounds,
895 is required, since these artificial sweeteners are largely metabolized in humans. Stability tests for
896 the metabolites in raw wastewater are also necessary for future studies. The use of artificial
897 sweeteners has been shown to be highly related to human activities (Buerge et al., 2009) and,
898 therefore, human consumption is considered as the major source of these substances in raw
899 wastewater; however, other sources, such as animal feedings, agriculture farms and industries, can
900 contribute to their presence in sewage systems (Kokotou et al., 2012).

901 Certain artificial sweeteners also showed a specific weekly pattern: in general higher loads
902 in influents (i.e. consumption) were observed during weekdays than during weekends (Kokotou et
903 al., 2013). This could be associated with more commuters during the weekday than the weekend in
904 the studied catchment. These previous studies together suggested that measuring artificial
905 sweeteners could be useful for the WBE approach to understand the population flow in a given
906 catchment. This concept of using human consumed chemicals, such as the artificial sweetener ACE,
907 to back-estimate the population size from a given wastewater sample was firstly attempted and
908 discussed by (Lai et al., 2011) and further refined using wastewater samples collected on the census
909 day and applying a Bayesian model (O'Brien et al., 2014). Importantly, with chemical-derived
910 population estimates, the robustness of the WBE data was improved, since the total methodological
911 uncertainty of the approach was reduced (Lai et al., 2015a, 2011).

912

913 **5.2. Nicotine**

914 Currently, nicotine and its metabolites have been used as population markers on two
915 occasions (Chen et al., 2014; Senta et al., 2015a). In the first case, the authors focused solely on
916 cotinine, whose loads varied only limitedly over one week and showed good correlation with the
917 size of the investigated populations (i.e., correlation coefficient = 0.981) (Chen et al., 2014).
918 However, geographical/cultural differences in tobacco use or fluctuations in the number of users
919 have been raised as potential flaws to the use of cotinine as population marker (Chen et al., 2014).
920 Moreover, consumption of tobacco could change due to tax and other tobacco-related policies,
921 which could affect the potential of nicotine and its metabolites as population markers. In the second
922 study (Senta et al., 2015a), cotinine and *trans*-3'-hydroxycotinine loads were used to estimate the
923 number of individuals contributing to the collected wastewater samples. Good agreement was found
924 between nicotine metabolite load population estimates and census data, suggesting that the method
925 is a viable approach to estimate the size of a population.

926

927 **5.3. Caffeine**

928 Caffeine and some of its major metabolites were recently tested as a population biomarkers.
929 Caffeine was one of the compounds included in the exploratory study to estimate population size
930 using samples collected on the census day and applying a Bayesian model (O'Brien et al., 2014). A
931 strong correlation between caffeine mass loads and population size was observed. In the second
932 study, generally good agreement between caffeine loads and hydrochemical parameters routinely
933 determined at the WWTPs was found (Rico et al., 2016). In another recent study, three major
934 caffeine metabolites: 1-methylxanthine, 7-methylxanthine and paraxanthine were tested together
935 with caffeine as possible population biomarkers (Senta et al., 2015a). These compounds fulfilled
936 some of the major requirements for an ideal biomarker - they are easily detectable and stable in
937 wastewater samples. However, their mass loads in wastewater did not completely reflect the human
938 excretion profile of caffeine, probably due to biases in caffeine pharmacokinetic data (see section
939 2.4 and **Table S2**) and additional sources of some metabolites and unconsumed caffeine. This
940 makes the possibility of using caffeine and/or its metabolites as biomarkers for population size
941 assessment rather difficult, at least without additional studies.

942

943 **5.4. Pharmaceuticals**

944 Concentrations and mass loads of pharmaceuticals in wastewater were used in the WBE field
945 for the estimation of population size only on three occasions (Lai et al., 2011; O'Brien et al., 2014;
946 Rico et al., 2016). The investigated compounds by Lai et al. (Lai et al., 2011) were atenolol (beta-
947 blocker), gabapentin (anti-convulsant), hydrochlorothiazide (diuretic), and venlafaxine (anti-
948 depressant). Atenolol was concluded to be the best option for this aim for the specific catchment. In
949 addition to the compounds selected by Lai et al., the same group also investigated carbamazepine
950 (antiepileptic), codeine, ibuprofen, paracetamol (analgesics), furosemide (diuretic), iopromide

951 (contrast medium), naproxen (anti-inflammatory) and salicylic acid (metabolite of acetylsalicylic
952 acid) and the measured loads were used in a collective model for the estimation of the population
953 size (O'Brien et al., 2014). By cross validating the data, the authors demonstrated that large
954 populations sizes could be estimated fairly accurately using the information of multiple chemical
955 mass loads. However, it could not be improved for small populations. In the work published by
956 (Rico et al., 2016) twelve human urine biomarkers were tested to estimate population size, six of
957 them being pharmaceuticals (hydrochlorothiazide, carbamazepine, codeine, naproxen, salicylic acid
958 and atenolol). However, by using these compounds, the population was under or overestimated
959 compared to the hydrochemical population, but they have good prospects if the appropriate data
960 sales are available.

961

962 **5.5. Endogenous compounds**

963 An alternative for estimating the population size in the catchment area of a WWTP relies on
964 monitoring influent wastewater for a biomarker linked to human metabolism. Chemicals involved
965 in endogenous metabolism avoid many of the problems encountered with xenobiotics, since their
966 association with human activities has a higher fidelity. Yet, their main problem is excessive intra-
967 and inter-individual variation in excretion. Biomarkers of endogenous origin derive from human
968 biochemical processes and undergo continuous urinary or fecal excretion. Several endogenous
969 biomarkers, which have been considered in the past or which have the potential to estimate the
970 population size more accurately (**Table S4**), are further discussed.

971 An important endogenous biomarker, widely used in clinical chemistry and with detailed
972 knowledge about its excretion, is creatinine (CR). A small portion of creatine (and
973 phosphocreatine), which is stored predominately in skeletal muscle, is continually converted to
974 form the endogenous anhydride, CR (a nitrogenous waste product cleared via the kidney); the rate
975 of conversion, in males for example, is about 1.6–1.7% per day. The major factors involved with

976 variability in CR output have been summarized by (Ryan et al., 2011). However, intra- and inter-
977 day CR excretion is not constant and daily excreted quantities can have high variance, being
978 strongly influenced by diet composition. In addition, CR is being increasingly used as a food and
979 nutritional supplement, adding yet another source of potential variation to CR excretion rates.
980 Although CR has been used in WBE studies as population marker (Brewer et al., 2012; Chiaia et
981 al., 2008), it was shown to be unstable in wastewater (completely decomposed within 24 h) (Chen
982 et al., 2014).

983 Another potential biomarker is coprostanol (CoP) that originates from gut microbial
984 metabolism, making up roughly 60% of the overall sterol content in human feces. CoP is poorly
985 absorbed from the gut (it does not undergo enterohepatic circulation) and is therefore fully excreted
986 in the feces. Since the 2000s, CoP has been used as anthropogenic marker in wastewater and to
987 gauge the degree of dilution of raw or treated wastewater in receiving surface water (Takada and
988 Eganhouse, 1998). However, CoP is excreted by various vertebrates in differing absolute and
989 relative quantities and it is sometimes difficult to distinguish between human and animal
990 contamination (Bull et al., 2002). Furthermore, CoP adsorbs substantially onto particulate matter
991 found in wastewater and was thus discarded as potential population marker (Chen et al., 2014).
992 Similar results were obtained for cholesterol (Chen et al., 2014); cortisol and androstenedione were
993 investigated, but rapidly degraded in wastewater (Chen et al., 2014).

994 Another example of biomarker relatively unique to human metabolism is 1-aminopropan-2-
995 one (1-aminopropanone: APR; 1-aminoketone). Through 1-aminopropan-2-ol, APR serves as a
996 precursor to vitamin B-12 (Fitzsimons and Belt, 2005). It is very water soluble and it is excreted via
997 urine, but in much lower daily quantities than CoP. However, it is sometimes found in wastewater
998 at levels higher than in urine, implicating potential *de novo* microbial formation in sewage
999 (Fitzsimons and Belt, 2005), whilst it could not be detected on other occasions (Singh and
1000 Gardinali, 2006).

1001 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, has also been investigated.
1002 Its excretion might be altered due to diseases (e.g., carcinoid tumors (Zuetenhorst, 2004)) and diet
1003 (i.e., some fruits and nuts (Feldman and Lee, 1985) and salt intake (Sharma et al., 1993).
1004 Furthermore, intra- and inter-individual variability in excretion has also been highlighted (Curtin et
1005 al., 1996). Results from wastewater analysis showed good correlation with census data and the
1006 authors considered it as a promising marker (Chen et al., 2014).

1007 Ammonium (NH_4^+) represents the major form in which ammonia (NH_3) is found in
1008 wastewater and originates from the breakdown of urea (Udert et al., 2006). It is mainly introduced
1009 via toilets (Butler et al., 1995) and it is routinely measured by WWTP as a water quality parameter.
1010 It is supposedly less affected by non-human sources compared to conventional parameters (e.g.,
1011 chemical or biological oxygen demand, total phosphorous) (van Nuijs et al., 2011b) and can
1012 potentially be measured online using ion-selective electrodes. Fluctuations in ammonium loads have
1013 been shown to link well to population dynamics (Been et al., 2014). Yet, its use to estimate absolute
1014 figures of the size of the *de facto* population might be undermined in rural areas due to the
1015 contribution of agricultural sources.

1016

1017 **5.6. DNA**

1018 Deoxyribonucleic acid (DNA) is a nucleic acid that carries most of the genetic instructions
1019 from all known living organisms and many viruses. DNA can be naturally shed into the
1020 environment through urine, feces, exudates or tissue residues. Compared to most of chemical
1021 compounds as a candidate of population biomarkers, DNA is much more stable and able to persist
1022 in the environment from month to hundred years depending on species (Prüfer et al., 2014;
1023 Thomsen and Willerslev, 2015). DNA biomarkers have been widely used in the field of medical
1024 diagnostics and biomedicine (Altintas and Tothill, 2013; Liu et al., 2011; Ralla et al., 2014; Wang et
1025 al., 2012). For WBE, DNA has a great potential to act as a population biomarker, not only because

1026 of its little affinity to other species in wastewater and constant excretion by humans, but also for its
1027 extreme stability and the possibility of being quantifiable Those robotic characteristics well meet
1028 the proposed criteria of a proper population biomarker candidate (Dejean et al., 2011; Thomsen and
1029 Willerslev, 2015).

1030 Typically, the changes of DNA component and structure such as DNA damage, repair and
1031 mutation could be used as biomarkers. Recently, a H2AX histone phosphorylation assay was
1032 developed as DNA damage biomarker for human population study, as it represents an early event in
1033 the cellular response against DNA double-strand breaks (Sánchez-Flores et al., 2015). However, to
1034 select a population biomarker for WBE uses, one of the crucial criteria is to screen human specific
1035 DNA. Wastewater is a complex matrix, which may contain DNA from various species such as
1036 plants, animals, and viruses. A recent study by Yang *et al* (Yang et al., 2015a, 2015b) has proposed
1037 to use community sewage sensors to identify human-specific mitochondrial DNA as a potential
1038 population biomarkers. In this study, human specific mitochondrial DNA associated with disease
1039 biomarkers (Liu et al., 2011; Tipirisetti et al., 2014) was amplified from wastewater by a
1040 specifically designed primer using quantitative real-time polymerase chain reaction (PCR) (Yang et
1041 al., 2015a). More importantly, the amplicons were detectable by an electrochemical biosensor based
1042 on a custom synthesized ferrocence intercalator as a signal transducer. The developed biosensors
1043 allow for the detection of single nucleotide variation and enable the potential of portable sensors for
1044 rapid identification of specific human biomarkers in wastewater.

1045

1046 6. CONCLUSIONS AND FUTURE PERSPECTIVES

1047 WBE is a rapidly developing scientific discipline with a strong transdisciplinary character. It
1048 has shown great progress, and opens up many possibilities for expanding its application to provide
1049 relevant information about lifestyle and public health.

1050 This review has outlined potential wastewater biomarkers of exposure or effect that could be
1051 used for future applications associated with lifestyle and wellbeing studies. However, it has also
1052 discussed limitations and highlighted that more research is needed, for various proposed
1053 biomarkers, before WBE can appropriately be applied. Moreover, several trends, needs and
1054 recommendations are indicated:

- 1055 - Human pharmacokinetic data (metabolism and urinary profile of excretion) are necessary to
1056 ensure that the candidate biomarker is formed in the body in a high proportion and is excreted
1057 mainly via urine. This information is highly relevant not only to back-calculate the
1058 consumption/exposure of a certain substance by a community, but also to distinguish the
1059 amount of a substance coming from human or other sources.
- 1060 - In-sample and in-sewer stability studies are needed for a better application in WBE. Stability
1061 tests are often performed in the laboratory, trying to reproduce the real conditions of
1062 temperature and sewage composition or in-sewer conditions. An alternative would be the use of
1063 *in-silico* tools to predict the stability of a compound in wastewater treatment processes. These
1064 models do not guarantee the formation of a biotransformation product, so it may be used as an
1065 indicator or a guide about the in-sewer stability of a residue and its potential adsorption (Reid
1066 2014). Sorption onto the solid particulate or the conjugation of the biomarkers must also be
1067 taken into account when assessing stability.
- 1068 - Source identification is needed to ensure that discharges from exogenous sources that might
1069 cause overestimation of the real amounts consumed are considered.

- 1070 - Cross validation of data (e.g. concentrations of pharmaceuticals in wastewater with bench-top
1071 sales) is recommended for all applications.
- 1072 - Multiple biomarkers for estimating the population size need to be set to allow for the
1073 normalization of the data. The development of portable biosensors may allow rapid estimation
1074 of the population contributing to the wastewater samples in the near future.
- 1075 - Regular monitoring of sewage for viruses based on similar DNA biosensors may give an early
1076 warning of a possible upcoming outbreak.
- 1077 - Omics approaches also hold promising and important roles in future developments and
1078 applications of endogenous biomarkers analysis in WBE.

1079

1080

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1102

1103

1104 **TABLES**

1105

1106 **Table 1.** Overview of the most relevant biomarkers used so far and potential biomarkers (for more
1107 details, please read the corresponding text and/or supporting information).
1108

Class	Parent compound	Biomarker/potential biomarker	WBE application	Reference
Illicit drugs	Cocaine	Benzoyllecgonine	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	Amphetamine	Amphetamine	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	Methamphetamine	Methamphetamine	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	MDMA	MDMA	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	THC/Cannabis	THC-COOH	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
Alcohol	Ethanol	Ethyl sulfate	YES	(Rodríguez-Álvarez et al., 2015)
Tobacco	Nicotine	Cotinine + trans-3'-hydroxycotinine	YES	(Castiglioni et al., 2015b)
Caffeine	Caffeine	See Table S1	NO	
NPS		See Table S1	NO	
Pesticides	20 pyrethroids	3-PBA	YES	(Rousis et al., 2016b)
	Permetrin, cypermethrin, cyfluthrin	cis-DCCA	YES	(Rousis et al., 2016b)
	Permetrin, cypermethrin, cyfluthrin	trans-DCCA	YES	(Rousis et al., 2016b)
Mycotoxins		See Table S2	NO	
Parabens		See Table S2	NO	
UV-filters		See Table S2	NO	
Plasticizers		See Table S2	NO	
Flame		See Table S2	NO	

retardants				
Pharmaceuticals	Atenolol	Atenolol	YES	(Baz-Lomba et al., 2016; van Nuijs et al., 2015)
	Citalopram	Citalopram	YES	(Baz-Lomba et al., 2016; van Nuijs et al., 2015)
	Carbamazepine	Carbamazepine	YES	(Baz-Lomba et al., 2016; van Nuijs et al., 2015)
	Diclofenac	Diclofenac	YES	(Baz-Lomba et al., 2016)
	Metformin	Metformin	YES	(van Nuijs et al., 2015)
	Valsartan	Valsartan	YES	(van Nuijs et al., 2015)
Benzodiazepines	Oxazepam	Oxazepam	YES	(Baz-Lomba et al., 2016)
Artificial sweeteners	Acesulfame	Acesulfame	YES	(Lai et al., 2015a)
Endogenous Compounds	Serotonin	5-HIAA	YES	(Rico et al., 2016)
	Ammonia	Ammonium	YES	(Been et al., 2014)

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1112 **FIGURE CAPTIONS**

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1114 **Figure 1.** Main requirements of a biomarker

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